ORIGINAL ARTICLE

FREQUENCY OF PANTON VALENTINE LEUCOCIDIN GENE IN *STAPHYLOCOCCUS AUREUS* FROM SKIN AND SOFT TISSUE INFECTIONS

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Background: *Staphylococcus aureus* harbouring Panton Valentine Leucocidin gene are emerging and spreading worldwide. PVL gene was first identified by Noel Panton and Francis Valentine in 1932 who explained its ability to lyse leucocytes and its main relationship with skin and soft tissue infections. In Pakistan only limited data is available on the frequency and molecular analysis of PVL gene positive *Staph aureus*. Therefore, this study was conducted to understand the clinical epidemiology of PVL positive *Staph aureus* in our setup. Objectives of the study was aimed to determine the frequency of PVL gene in *Staph aureus* obtained from pus samples from skin and soft tissue infections from various departments; indoor and outdoor of a tertiary care hospital of Lahore. **Methods:** 384 *Staph aureus* isolates from skin and soft tissue infections were selected from both indoor and outdoor departments of hospital. After identification by phenotypic methods, they were processed by PCR using luk-F and luk-S primers for the detection of PVL gene. **Results:** 186 out of 384 *Staph aureus* isolates were positive for PVL gene. Overall frequency of PVL gene was 49%. Frequency of PVL gene in *Staph aureus* was 44.9% in males and 53.5% in females. The highest frequency of PVL gene was detected in paediatric age group. A large majority of positive isolates were from pus samples other than swabs and from the general surgery department. They mostly belong to indoor with indoor outdoor ratio of approximately 2:1. Frequencies of PVL gene in MRSA and MSSA were 51% and 44% respectively. Frequency of PVL gene was found to be high in Ciprofloxacin sensitive, Gentamicin sensitive, Erythromycin resistant and Fusidic acid resistant isolates. **Conclusion:** Almost half of *Staph aureus* isolates were found PVL positive. They were mostly multidrug resistant came from indoor setup. This situation is very alarming so, there is a need to adopt strict infection control policies in the hospitals to limit the widespread and injudicious use of antibiotics. There is also a need to apply PVL positive *Staph aureus* treatment to the effected individuals which involve not only antibiotics but also the decolonization of effected individuals and their close contacts.

Keywords: Penton Valentine Leucocidin; Methicillin Resistant Staph aureus; Methicillin Sensitive Staph aureus; Multidrug Resistant; Polymerization Chain Reaction; Skin and Soft Tissue Infections

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INTRODUCTION

Staphylococci are gram-positive cocci that are microscopically seen as sporadic, grapelike groups. They are nonmotile and catalase-positive microscopic organisms. They are present as commensals in various parts of body.[1] *S. aureus* is the most widely recognized microbe that causes skin and soft tissue diseases in both children and adults.[2] *Staph aureus* also cause life-threatening infections, for example, pneumonia and endocarditis.[3] Pathogenic factors include surface proteins, toxins like PVL (Panton Valentine Leucocidin) toxin and various enzymes.[4] PVL gene is acquired by *Staph aureus* by viruses called Prophages which carry various genes between bacteria.[5]

Panton Valentine Leucocidin gene produces PVL toxin that destroys the outer membranes of white blood cells by the combine action of 2 secretory proteins named S and F by making pores in the membranes of cell and causing leakage of cellular contents through that pores resulting in cell death.[6] Clinical sequalae of PVL positive *Staph aureus* infections are more severe than PVL negative *Staph aureus* infections.[7] PVL-positive *S. aureus* is quickly arising across the world and it has a prominent place in global public health setting. Countries in western and central Africa have a higher frequency of 57%, raising serious apprehensions regarding the spread of virulent PVL-positive MRSA strains.[8] Data from different countries in the world is as follows: 12.8% in China[9], 30% in Germany,[10] 1.8% in Ireland,[11] 0.9% in Korea,[12] 11.6% in Singapore,[13] 4% in

Turkey,[14] and 97% in the United States[15]. In South Asia, the rates of PVL gene in *Staph aureus* in various studies range from 6% in Iran to 62.85 in India.[16] Limited work has been done in Pakistan on PVL gene. In a study from Malakand division of Khyber Pakhtunkhwa, overall frequency of PVL gene in *Staph aureus* (sample size=45) was reported 49%.[17] This frequency is very high as it indicates that almost half of *Staph aureus* that were collected from skin and soft tissue infections in hospital had PVL gene. If this is the ratio in whole of Pakistan than it is an alarming sign for our society and healthcare settings. To assess this and to study antimicrobial pattern of the PVL positive and negative isolates, we performed this study in another region of Pakistan (Lahore, Punjab) on a large sample size of 384 collected from various departments of a tertiary care hospital (Shaikh Zayed Hospital Lahore).

MATERIAL AND METHODS

A total of 384 was the size of sample that was estimated by using 95% confidence level and 5% margin of error with expected frequency of PVL gene among *Staph aureus* positive cases being 49%. Only pus samples from skin and soft tissue infections were included. Only one pus sample per patient was taken. Data regarding name, age, gender, department, indoor or outdoor and history of sample was noted on standardized Proforma. Pus samples were cultured and *Staph aureus* were identified by colony morphology, gram staining, catalase positive, coagulase positive, and DNAs positive results performed according to laboratory SOPs and stored in solution made up of nutrient broth and glycerine. Antimicrobial sensitivity testing was carried out by manual AST method according to CLSI guidelines. Following drugs were used; Cefoxitin, Erythromycin, Ciprofloxacin, Gentamycin, Fusidic acid, Penicillin, Vancomycin, and Linezolid.

Molecular analysis for the detection of PVL gene in *Staph aureus* was carried out in Molecular Microbiology Laboratory, Shaikh Zayed Hospital Lahore. Genetic analysis includes DNA extraction, PCR, Gel Electrophoresis and Gene sequencing. DNA extraction was done with the help of DNA extraction kit from Sacace Biotechnologies (REF: K-2/C/100, LOT: 1602011). PVL gene sequence was submitted to NCBI were studied and then primers were designed against a sequence from NCBI whose accession number was confirmed through gene bank. Following primers were used for PVL gene PCR; FORWARD PRIMER: ATCATTAGGTAAAATGTCTGGACATGATCCA REVERSE PRIMER: GCATCAAGTGTATTGGATAGCAAAAGC. Gene size=433BP. Stock solution of primers was made by adding 300 microliter water in lipolyzed primers. Thus, 100pm solution of primers per microliter was obtained. Then by adding 90µl dH2O in 10µl of primers. For Gel Electrophoresis 1% agarose gel was used. The presence of Panton Valentine Leucocidin gene was also checked by using the above described primers. The PCR reaction mixture details are given in Table-1. Thermo cycling conditions consisted of one cycle of 5 minutes of 94°C followed by 35 cycles of amplification each consisting of three steps: thirty seconds at 94 °C; thirty seconds at 57°C and thirty seconds at 72 °C, followed by a final extension step of 10 minutes at 72 °C. PCR was performed using a Triple Master Mix Thermo cycler (Eppendorf, USA).

Before running research samples, we sent one PVL positive PCR sample for sequencing and confirmation to Advance Bioscience International and it was used as control for PVL gene.

RESULTS

Results were analysed by using latest version of Statistical Package of Social Sciences. Chi-square test was applied. A total of 1404 pus samples were received for culture sensitivity testing in Microbiology Laboratory, Shaikh Zayed hospital, Lahore during a period of one year. From these pus samples 384 Staph aureus were isolated and selected for research purpose. Among these pus samples, 868 were from male patients and 536 were from female patients. The frequency of *Staph aureus* was 26% from male pus samples and 29% from female pus samples. Frequency of PVL gene in *Staph aureus* was 44.9% in males and 53.5% in females. The frequency of MRSA was 65% in males and 74% in females. Total number of pus samples from indoor were 938 and outdoor were 466. The frequency of *Staph aureus* was 26% from indoor samples and 30% from outdoor samples. The percentage of PVL gene in *Staph aureus* was 49.3% from indoor and 47% from outdoor samples and percentage of PVL gene in *Staph aureus* was marginally high in indoor cases. In indoor cases, MRSA were 72% and MSSA were 28%. Among OPD cases MRSA were 62% and MSSA were 38%. For study purpose and data analysis, patients were divided into 5 age groups with age gap of 15 years. This data showed that maximum number of *Staph aureus* was isolated from group 2 (age: 15–30 years) but PVL gene frequency was high in paediatric age group. The relationship between age group and PVL gene was not significant (p-value> 0.05). Type of specimen included in research were wound swab 49%, ear swab 5%, nasal swab 1%, skin lesions 32% and pus samples other than swabs was 12.5%. Maximum number of *Staph aureus* were isolated from wound swab while PVL gene frequency

was highest in pus samples from various other sites of body, 52%.

The relationship of *Staph aureus* from various departments with PVL gene and Methicillin resistance was not significant (*p*-value>0.05). Maximum number of *Staph aureus* and MRSA were from Surgery department with 55.5% PVL gene frequency. Maximum number of MRSA were found in Ciprofloxacin resistant cases while PVL gene frequency was high in Ciprofloxacin sensitive MRSA (p value insignificant). Similarly, maximum number of MRSA were found in Erythromycin resistant cases while PVL gene frequency was high in Erythromycin intermediate MRSA. The maximum number of MRSA were found in Gentamycin resistant cases while PVL gene frequency was high in Gentamycin sensitive MRSA. Maximum number of MRSA were found in Fusidic acid resistant cases while PVL gene frequency was high in Fusidic acid sensitive MRSA. Among 384 *Staph aureus* 75% were Multi Drug Resistant (MDR). Multidrug resistant isolates included those that were resistant to 3 or more different groups of drugs. 49.3% MDR *Staph aureus* and 46% non-MDR *Staph aureus* carried PVL gene. Among 384 *Staph aureus* 68.5% were MRSA and half of these MRSA were PVL +ve (50%).

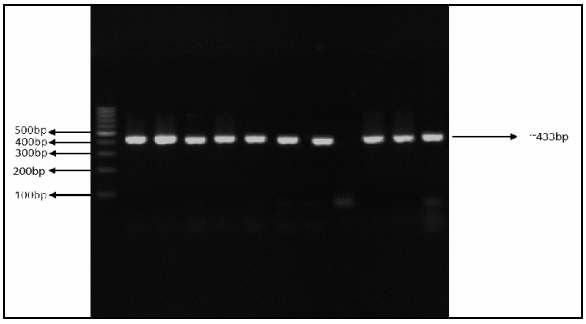

Figure-1: PVL gene on gel electrophoresis

Table-1: Reaction mixture of PCR

Name of reaction components	Volume (µl)
PCR buffer	2.5
MgCl2 (25mm)	2
dNTPs (10mm)	2.5
Primer-F (10pm)	1
Primer-R (10pm)	1
Taq-Polymerase (5U/µl)	0.5
H2O (distilled)	13
PCR Mix/Reaction	22.5
DNA	2.5 each
Total	25/per reaction

Table-2 Distribution of pus samples, *Staph aureus* and PVL gene by gender

Variables	Total	Males	Females
Pus samples	1404	868 (62%)	536 (38%)
Staph aureus	384 (27%)	227 (26%)	157 (29%)
PVL gene +VE	186 (48%)	102 (45%)	84 (53.5%)

Table-3: Distribution of pus samples, *Staph aureus* and PVL gene in OPD & IPD

Variables	Total	Indoor	Outdoor
Pus samples	1404	938 (67%)	466 (33%)
Staph aureus	384 (27%)	245 (26%)	139(30%)
PVL gene+VE	186 (48%)	121 (49%)	65 (47%)

DISCUSSION

Staphylococcus aureus is a very dominant pathogen that causes infections of mild to severe range in both hospital and community settings. *Staph aureus* carrying PVL gene has attained global attention due to its high virulence and pathogenicity. It causes not only skin and soft tissue infections but also life-threatening infections like haemorrhagic pneumonia. It causes infections in children and healthy young adults with no previous risk factors and contributes to high morbidity and mortality rate.[21] So, we worked on *Staph aureus* to understand its epidemiology and genetics. Our investigations concluded that in our setup almost half of *Staph aureus* had PVL gene and they should be given PVL+ve *Staph aureus* treatment. Data of our investigation shows that isolation of *Staph aureus* and frequency of not only PVL gene but also, MRSA was higher in female patients than male patients. These results are different from other research works, for example, in 2009 a research was conducted in Iran that showed 41.67% PVL gene in females and 58.3% in males.[18] MJ Ellington *et al*. research on PVL gene in England and Wales in 2009 showed a high prevalence of PVL gene and MRSA in males as compared to females. 99 females and 147 males were PVL positive among 275 MRSA isolates (the gender of 29 individuals was not available).[19] In recent investigation, most *Staph aureus* were isolated from pus samples from OPD rather than IPD but PVL gene frequency was higher in indoor *Staph aureus* isolates than outdoor probably due to increasing frequency of MRSA from indoor than outdoor, as it is said that PVL gene frequency is usually high in MRSA than MSSA.[19] High frequency of MRSA from indoor may also be due to the reason that there is more intense use of antibiotics in hospitals as compared to community settings. According to current research data, PVL gene frequency was higher in the paediatric age group. Our results are in accordance with other research works which revealed high PVL gene burden in children than adults and old age group. In a study from India, PVL gene was high in children having age less than 14 years old.[20] It has been seen that PVL+ve *Staph aureus* has a strong association with skin and soft tissue infections, in fact, most studies have proved that PVL gene frequency is high in pus samples from SSTIs as compared to blood, urine or sputum samples.[4] Samples were collected from 17 different

hospital departments both from indoor and outdoor. Maximum samples were from surgery (34%) followed by orthopaedics (23%), medicine (7%), paediatric surgery (7%), dermatology (6.8%), plastic surgery (5.2%) and burn center (1%). MRSA and PVL gene frequency was higher in surgery than orthopaedics and so on, according to their total percentage. A similar distribution of PVL gene among various hospital departments has been reported from Nepal, 82.2% from surgery, 6.3% from orthopaedic surgery, 6% from burn center and 5% from other departments [22] and also from India. [20]

Antimicrobial sensitivity of *Staph aureus* isolates was tested and among 384 samples, 68.5% were MRSA and 31.5% were MSSA. Among MRSA 50.6% have PVL gene while in MSSA 43.8% have PVL gene. Suberna Roy *et al*. from India have reported 85.1% PVL gene in MRSA and 48.8% in MSSA which indicated a higher prevalence of PVL gene than my findings[16]. Another study conducted in Pakistan in 2016 reported 44% MRSA and 55% MSSA while PVL gene frequency in MRSA was 31% and in MSSA it was 18%[17] indicated a lower prevalence of PVL gene than my findings. So, current research work indicates that the frequency of both MRSA and PVL may be increasing in our setup with the passage of time. This may be due to unnecessary and excessive use of antibiotics in our setup that is making isolates more antibiotic resistant and causing poor infection control and selection of resistant isolates in the community. It has been assessed that PVL gene frequency is usually high in Ciprofloxacin sensitive *Staph aureus.*[19] We found high percentage of PVL gene in Ciprofloxacin sensitive *Staph aureus* but the difference of percentage was not so high and it was also not statistically proved. In current investigation, Ciprofloxacin resistance in *Staph aureus* was 72% while it had been reported as low as less than 3% from England and Wales.[4] These results reflect that prevalence of Ciprofloxacin resistance and PVL gene varies greatly between geographical locations and populations. Our investigations show that more than half of *Staph aureus* were resistant to Erythromycin and PVL gene frequency was high in Erythromycin resistant MRSA. These results are in accordance with the results of research from Nepal.[22] Current results show that 38% of *Staph aureus* were resistant to Gentamycin and frequency of PVL gene was high in Gentamycin sensitive MRSA and this result was statistically proved. Gentamycin resistance in *Staph aureus* was reported 20% in research from England.[4]

CONCLUSION

The overall result of this study is the observation of the high rate of PVL positive *Staph aureus* in our setup and frequency of the PVL gene is higher among pus samples which indicates a possible key role of PVL gene in the pathogenesis of pyogenic infections especially skin and soft tissue infections. This study also concluded that almost half of *Staph aureus* in our setup carry PVL gene. Since PVL gene is a very important virulence factor, so, it is recommended that PVL positive cases should be given PVL positive *Staph aureus* treatment which is different from PVL negative cases and preventive measures should be applied for further spread of these isolates.

Ethical approval to conduct the study was obtained from the Institutional Review Board (IRB), Federal Postgraduate Medical Institute Shaikh Zayed Hospital Lahore. Consent of patients was not required as samples were taken as a routine part of care.

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AUTHORS' CONTRIBUTION

AA: Literature search, write-up, data collection, analysis. MI: Conceptualization of study design. CL: Data analysis and interpretation. HG, SZ: Proof reading. AS: Data collection. AY: Data collection, analysis, genetic analysis.

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